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OPTIMIZATION OF LIGAND AFFINITY FOR RNA TARGETS USING MASS SPECTROMETRY

FIELD OF THE INVENTION

The present invention is related to mass spectrometry methods for detecting binding interactions of ligands to substrates and in particular to methods for
5 determining the mode of binding interaction of legends to substrates.

BACKGROUND OF THE INVENTION

Drug discovery has evolved from the random screening of natural products into a combinatorial approach of
10 designing large numbers of synthetic molecules as potential bioactive agents (ligands, agonists, antagonists, and inhibitors). Traditionally, drug discovery and optimization have involved the expensive
15 and time-consuming process of synthesis and evaluation of single compounds bearing incremental structural changes. For natural products, the individual components of extracts had to be painstakingly separated into pure

constituent compounds prior to biological evaluation. Further, all compounds had to be analyzed and characterized prior to in vitro screening. These screens typically included the evaluation of candidate compounds

5   for binding affinity to their target, competition for the ligand binding site, or efficacy at the target as determined via inhibition, cell proliferation, activation or antagonism end points. Considering all these facets of drug design and screening that slow the process of

10   drug discovery, a number of approaches to alleviate or remedy these matters, have been implemented by those involved in discovery efforts.

The development and use of combinatorial chemistry has radically changed the way diverse chemical compounds

15   are synthesized as potential drug candidates. The high-throughput screening of hundreds of thousands of small molecules against a biological target has become the norm in many pharmaceutical companies. The screening of a combinatorial library of compounds requires the

20   subsequent identification of the active component, which can be difficult and time consuming. In addition, compounds are usually tested as mixtures to efficiently screen large numbers of molecules.

A shortcoming of existing assays relates to the

25   problem of "false positives." In a typical functional assay, a false positive is a compound that triggers the assay but which compound is not effective in eliciting the desired physiological response. In a typical physical assay, a false positive is a compound that

30   attaches itself to the target but in a non-specific manner (e.g. non-specific binding). False positives are particularly prevalent and problematic when screening higher concentrations of putative ligands because many compounds have non-specific affects at those

35   concentrations. Methods for directly identifying

compounds that bind to macromolecules in the presence of those that do not bind to the target could significantly reduce the number of "false positives" and eliminate the need for deconvoluting active mixtures.

5   In a similar fashion, existing assays are also plagued by the problem of "false negatives," which result when a compound gives a negative response in the assay but the compound is actually a ligand for the target. False negatives typically occur in assays that use

10   concentrations of test compounds that are either too high (resulting in toxicity) or too low relative to the binding or dissociation constant of the compound to the target.

When a drug discovery scientist screens

15   combinatorial mixtures of compounds, the scientist will conventionally identify an active pool, deconvolute it into its individual members, and identify the active members via re-synthesis and analysis of the discrete compounds.   In addition to false positives and false

20   negative, current techniques and protocols for the study of combinatorial libraries against a variety of biologically relevant targets have other shortcomings. These include the tedious nature, high cost, multi-step character, and low sensitivity of many screening

25   technologies. These techniques do not always afford the most relevant structural and binding information, for example, the structure of a target in solution and the nature and the mode of the binding of the ligand with the receptor site. Further, they do not give relevant

30   information as to whether a ligand is a competitive, noncompetitive, concurrent or a cooperative binder of the biological target's binding site.

The screening of diverse libraries of small molecules created by combinatorial synthetic methods is a

35   recent development that has the potential to accelerate

the identification of lead compounds in drug discovery. Rapid and direct methods have been developed to identify lead compounds in drug discovery involving affinity selection and mass spectrometry. In this strategy, the receptor or target molecule of interest is used to isolate the active components from the library physically, followed by direct structural identification of the active compounds bound to the target molecule by mass spectrometry. In a drug design strategy, structurally diverse libraries can be used for the initial identification of lead compounds. Once lead compounds have been identified, libraries containing compounds chemically similar to the lead compound can be generated and used to develop a structural activity relationship (SAR) in order to optimize the binding characteristics of the ligand with the target receptor.

One step in the identification of bioactive compounds involves the determination of binding affinity and binding mode of test compounds for a desired biopolymeric or other receptor. For combinatorial chemistry, with its ability to synthesize, or isolate from natural sources, large numbers of compounds for in vitro biological screening, this challenge is greatly magnified. Since combinatorial chemistry generates large numbers of compounds, often isolated as mixtures, there is a need for methods which allow rapid determination of those members of the library or mixture that are most active, those which bind with the highest affinity, and the nature and the mode of the binding of a ligand to a receptor target.

An analysis of the nature and strength of the interaction between a ligand (agonist, antagonist, or inhibitor) and its target can be performed by ELISA (Kemeny and Challacombe, in *ELISA and other Solid Phase*

Immunoassays: Theoretical and Practical Aspects; Wiley, New York, 1988), radioligand binding assays (Berson and Yalow, Clin. Chim. Acta, 1968, 22, 51-60; Chard, in "*An Introduction to Radioimmunoassay and Related Techniques,*" Elsevier press, Amsterdam/New York, 1982), surface-plasmon resonance (Karlsson, Michaelsson and Mattson, *J. Immunol. Methods*, 1991, 145, 229; Jonsson et al., *Biotechniques*, 1991, 11, 620), or scintillation proximity assays (Udenfriend, Gerber and Nelson, Anal. *Biochem.*, 1987, 161, 494-500). Radio-ligand binding assays are typically useful only when assessing the competitive binding of the unknown at the binding site for that of the radio-ligand and also require the use of radioactivity. The surface-plasmon resonance technique is more straightforward to use, but is also quite costly. Conventional biochemical assays of binding kinetics, and dissociation and association constants are also helpful in elucidating the nature of the target-ligand interactions but are limited to the analysis of a few discrete compounds.

A nuclear magnetic resonance (NMR)-based method is described in which small organic molecules that bind to proximal subsites of a protein are identified, optimized, and linked together to produce high-affinity ligands (Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science*, **1996**, 274, 5252, 1531). The approach is called SAR by NMR because structure-activity relationships (SAR) are obtained from NMR. This technique has several drawbacks for routine screening of a library of compounds. For example, the biological target is required to incorporate a ^{15}N label. Typically the nitrogen atom of the label is part of amide moiety within the molecule. Because this technique requires deshielding between nuclei of proximal atoms, the ^{15}N

label must also be in close proximity to a biological target's binding site to identify ligands that bind to that site. The binding of a ligand conveys only the approximate location of the ligands. It provides no

5 information about the strength or mode of binding.

Therefore, methods for the screening and identification of complex target/ligand binding are greatly needed. In particular, new methods are needed for the identification of the strength and mode of

10 binding of a ligand to its intended target.

SUMMARY OF THE INVENTION

This invention provides for methods and processes for identifying weak binding ligands for a target

15 molecule. Ligands are selected that have an affinity for the target molecule that is equal to or greater than a baseline affinity. This can be accomplished according to one embodiment of the invention by utilizing a mass spectrometer and selecting a standard ligand that forms a

20 non-covalent binding complex with the target molecule. An amount of the standard ligand is mixed with an excess amount of the target molecule such that unbound target molecule is present in the mixture. This mixture is introduced into the mass spectrometer and the operating

25 performance conditions of the mass spectrometer are adjusted such that the signal strength of the standard ligand bound to the target molecule is from about 1% to about 30% of the signal strength of unbound target molecule. At least one further ligand is introduced into

30 a test mixture of the target molecule and the standard ligand and this test mixture is introduced into the mass spectrometer. Any complexes of the further ligand and the target wherein the ligand has greater than baseline affinity for the target molecule is identified by

discerning the signals that have a signal strength greater than the background noise of the mass spectrometer.

5   The invention further provides for methods and processes for selecting those members of a group of compounds that can form a non-covalent complex with a target molecule and where the affinity of the members for the target molecule is greater than a baseline affinity. This can be accomplished by utilizing a mass spectrometer

10   and selecting a standard compound that forms a non-covalent binding complex with the target molecule. An amount of the standard compound is mixed with an excess amount of the target molecule such that unbound target molecule is present in the mixture and the mixture is

15   introduced into the mass spectrometer. The operating performance conditions of the mass spectrometer are adjusted such that the signal strength of the standard compound bound to the target molecule is from about 1% to about 30% of signal strength of unbound target molecule.

20   Next a sub-set of the group of compounds is introduced into a test mixture of the target molecule and standard compound and this test mixture is introduced into the mass spectrometer. Those members of the sub-set of compounds that form complexes with the target with an

25   affinity greater than baseline are identified by discerning those signals that have a signal strength greater than the background noise of the mass spectrometer. The individual members are then identified by their respective molecular masses.

30   The invention further includes methods and processes for determining the relative interaction between at least two ligands with respect to a target substrate. This is accomplished by mixing an amount of each of the ligands with an amount of the target substrate to form a mixture.

35   The mixture is then analyzed using mass spectrometry to

determine the presence or absence of a ternary complex corresponding to simultaneous binding of two of the ligands with the target substrate. The absence of a ternary complex in the mixture indicates that binding of

5   the ligands to the target is competitive while the presence of a ternary complex indicates that binding of the ligands is other than competitive.

The invention further includes methods and processes for determining the binding interaction of ligands to a

10   target substrate. This is accomplished by mass spectrometry analysis of the mixture as described to determine if the binding is other than competitive followed by determination of the ion abundance of i) a ternary complex present in the mixture, ii) a first

15   binary complex corresponding to the adduction of a first ligand with the target substrate; iii) a second binary complex corresponding to the adduction of a second ligand with the target substrate; and iv) target substrate unbound or not complexed with either of the first or

20   second ligands. The absolute ion abundance of the ternary complex is compared to the sum of the relative ion abundance of the binary complexes which contribute to the formation of the ternary complex. The relative ion abundance of one of the contributing binary complexes is

25   calculated by multiplying the absolute ion abundance of the first binary complex with the relative ion abundance of the second binary complex with respect to the unbound target substrate. The relative ion abundance of the second binary complex is calculated by dividing that

30   binary complex' absolute ion abundance by the absolute ion abundance of the unbound target. Similarly, the relative ion abundance of the other contributing binary complex is calculated by multiplying the absolute ion abundance of the second binary complex with the relative

35   ion abundance of the first binary complex.

If the absolute ion abundance of the ternary complex is equal to the sum of the relative ion abundances of the contributing binary complexes this indicates concurrent binding interaction of the ligands to the target

5   substrate. If the absolute ternary complex ion abundance is greater this indicates cooperative binding interaction, and if lesser this indicates competitive binding interaction.

The invention further includes methods and processes

10   for determining the relative proximity of binding sites of a first and a second ligand on a target substrate. This can be accomplished by exposing the target substrate to a mixture of the second ligand and a plurality of derivative compounds of the first ligand. Each of the

15   first ligand derivatives has the chemical structure of the first ligand and at least one substituent group pending from it or if the first ligand includes a ring within its structure, derivatives of the ligand can include expansion of contraction of that ring. This

20   mixture is analyzed by mass spectrometry to identify a first ligand derivative that inhibits the binding of the second ligand to the target substrate or visa versa, i.e. binds competitively with the second ligand as determined by the absence of a ternary complex corresponding to the

25   simultaneous complexation of the first ligand derivative and the second ligand with the target.

This invention further provides for methods and processes for determining the relative orientation of a first ligand to a second ligand when these ligands are

30   bound to a target substrate. This is accomplished by exposing the target substrate to a mixture of the second ligand and a plurality of derivative compounds of the first ligand. Each of the first ligand derivatives has the chemical structure of the first ligand and a

35   substituent group pending therefrom. The mixture is

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analyzed by mass spectrometry to identify a first ligand derivative that inhibits the binding of the second ligand to the target substrate or visa versa, i.e. binds competitively with the second ligand as determined by the absence of a ternary complex corresponding to the simultaneous complexation of the first ligand derivative and the second ligand with the target.

This invention further provides for a screening method for determining compounds that have binding affinity to a target substrate. This is accomplished using mass spectrometry to identifying two ligands that bind to a target non-competitively in a mixture of the ligands and target substrate. These two ligands are then concatenated to form another ligand that has greater binding affinity for the target substrate than either of the two ligands.

The invention further includes methods and processes for modulating the binding affinity of ligands for a target molecule. This is accomplished by selecting a first ligand fragment and a second ligand fragment and then exposing a target molecule to these ligand fragments. The target molecule exposed to the ligand fragments is then interrogated in a mass spectrometer to identify binding of the ligand fragments to the target molecules. The ligand fragments are concatenated together in a structural configuration that improves the binding properties of the fragments for the target molecule.

The invention further includes methods and processes for refining the binding of ligands to target molecules. This is accomplished by selecting first and second virtual fragments of a ligand followed by virtually concatenating the selected ligand fragments together *in silico* to form a 3D model of the concatenated ligand

fragments. This 3D model of the concatenated ligand fragments is then positioned *in silico* on a 3D model of the target molecule. The various *in silico* positions of the 3D model of the concatenated ligand fragments on the

5 *in silico* 3D model of the target molecule are scored. Using the results of the scoring, the *in silico* position of the 3D model of the concatenated ligand fragments on the *in silico* 3D model of the target molecule is refined.

In a preferred embodiment of this method, real ligand

10 fragments corresponding to the virtual ligand fragments are concatenated together to covalently join these ligand fragments into a new molecule. The new molecule is mixed with a target molecule and the mixture interrogated in the mass spectrometer for binding of the new molecule to

15 the target molecule.

In each of the above methods and processes, in a preferred embodiment, an electrospray mass spectrometer is utilized. Preferred electrospray ionization is accomplished by Z-spray, microspray, off-axis spray or

20 pneumatically assisted electrospray ionization. Further countercurrent drying gas can be used. Preferred mass analyzers for use in identifying the complexes are quadrupole, quadrupole ion trap, time-of-flight, FT-ICR and hybrid mass detectors. The preferred method of

25 measuring signal strength is by the relative ion abundance. The mass spectrometer can also include a gated ion storage device for effecting thermolysis of the test mixtures within the mass spectrometer.

Adjustment of the mass spectrometer operating

30 performance conditions would include adjustment of the source voltage potential across the desolvation capillary and a lens element of the mass spectrometer. This is best monitored by ion abundance of free target molecule. Adjustment of the mass spectrometer operating conditions

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further can include adjustment of the temperature of the desolvation capillary and adjustment of the operating gas pressure with the mass spectrometer downstream of the desolvation capillary.

5   In a preferred embodiment, adjustment of the operating performance conditions of the mass spectrometer is effected by adjustment of the voltage potential across the desolvation capillary and a lens element to generate an ion abundance of the ion from a complex of standard

10   ligand with the target of from about 1% to about 30% compared to the abundance of the ion from the target molecule. A more preferred range of abundance of the complex of standard ligand with target to the abundance of the ion from the target molecule is from about 10% to

15   about 20%.

       Preferred for standard ligands are those ligands having a baseline affinity for the target of about 10 to about 100 millimolar. Particularly preferred are standard ligands having a baseline affinity for the

20   target molecule of about 50 millimolar as expressed as a dissociation constant. Particularly preferred for standard ligands for nucleic acid targets are amines, primary, secondary or tertiary, amino acids, and nitrogen containing heterocycles with ammonium being the most

25   preferred. Particularly preferred for standard ligands for peptides are esters, phosphates, borates, amino acid and nitrogen containing heterocycles.

       The target molecule can be one of various target molecules including RNA, DNA, proteins, RNA-DNA duplexes,

30   DNA duplexes, polysaccharides, phospholipids and glycolipids. Preferred are nucleic acids and proteins with RNA being particularly preferred as a target molecule.

       Various RNA molecules are useful as the target.

35   Preferred RNA target molecules are those that are

fragments of larger RNA molecules including those being from about 10 to about 200 nucleotides in length. A more preferred RNA target is RNA of from about 15 to about 100 nucleotides in length including those having secondary

5   and ternary structure.

Preferred ligand molecules include those having a molecular mass of less than about 1000 Daltons and fewer that 15 rotatable bonds, i.e., covalent bonds linking one atom to a further atom in the molecule and subject to

10   rotation of the respective atoms about the axis of the bond. More preferred ligands molecules include those having a molecular mass of less than about 600 Daltons and fewer than 8 rotatable bonds. Even more preferred ligand molecules include those have a molecular mass of

15   less than about 200 Daltons and fewer than 4 rotatable bonds. Further preferred ligands include those having no more than one sulfur, phosphorous or halogen atom.

The ligands can comprise members of collection libraries. Preferred collection libraries include

20   historical repositories of compounds, collections of natural products, collections of drug substances or intermediates for such drug substances, collections of dyestuffs, commercial collections of compounds or combinatorial libraries of compounds. A preferred

25   collection for selecting ligands can contain various numbers of members with libraries of from 2 to about 100,000 being preferred.

30   **BRIEF DESCRIPTION OF THE DRAWINGS**

Figure 1 is a schematic representation of a mass spectrometer employing an electospray ion source.

Figure 2 is a mass spectrum showing binding of a

small molecule ligand (2-amino-4-benzylthio-1,2,4-triazole) to a 27-mer fragment of bacterial 16S A-site ribosomal RNA and ammonium as standard ligand.

5   Figure 3 is a mass spectrum showing competitive displacement of glucosamine from the 16S RNA fragment by Ibis-326732.

Figure 4 is a mass spectrum showing the concurrent binding of 2-DOS and 3,5-diamino-1,2,4-triazole to the 16S RNA fragment.

10   Figure 5 is a table of particular amines and carboxylic acids that were conjugated at the R group in all combinations to form a library of amide linked compounds. The amide linked compounds were analyzed by mass spectroscopy to determine their binding affinity to 15   16S RNA fragment.

Figure 6 is a mass spectrum showing the binding of a piperazinyl small molecule IBIS-326611 from the amide library to 16S RNA fragment.

Figure 7 is a mass spectrum showing the binding to 20   16S RNA fragment of another piperazinyl small molecule IBIS-326645 from the amide library.

Figure 8 is a mass spectrum showing the enhanced binding to the 16S RNA fragment of concatenated compound IBIS-271583, derived from the structures of IBIS-326611 25   and IBIS-326645 and sharing the common piperazine moiety of the two parent compounds. The concatenated compound has greater affinity for 16S than either parent compound.

Figure 9 is a schematic representation of the binding of triazole and 2-deoxystreptamine ligands 30   binding at their respective binding sites on the target 16S RNA fragment and a concatenated compound derived from the two ligands.

DETAILED DESCRIPTION OF THE INVENTION

The methods of the present invention are useful for the detection, evaluation and optimization of ligands to

5   targets especially biological targets. The methods and processes of the invention utilize mass spectrometry as the primary tool to accomplish this. The detection and evaluation of the different binding modes of noncovalently bound ligands to a target are useful for

10   advancing the structure activity relationship (SAR) and for designing ligands with higher binding affinities for their given target sites.

Mass spectrometry has been used to afford direct and rapid methods to identify lead compounds and to study the

15   interactions between small molecules and biological targets. An advantage of mass spectrometry in identifying lead compounds is the sensitivity of the detection process. Small molecules (ligands) which bind to a target through weak noncovalent interactions, may be

20   missed through conventional screening assays. These noncovalent ligand:target complexes, however, are readily detected by mass spectral analysis using the methods and processes of the invention.

These small molecules include both tight and weak

25   binding ligands that bind to a particular target. In both collections of compounds and in biological samples, tight binding ligands can be present in very low concentrations relative to the weaker binding ligands. A tight binding ligand may be part of a very large library

30   of compounds (e.g. a combinatorial library) or may be present in trace amounts of a tissue extract. In both cases, there is usually a much higher concentration of weaker binding ligands relative to the tight binding ligands.

A tight or a weak binding ligand can bind to a target by a noncovalent bond. These noncovalent interactions include hydrogen-bonding, electrostatic, and hydrophobic contacts that contribute to the binding

5   affinity for the target. The difference between a tight and weak binding ligand is relative, a tight binding ligand has a stronger interaction between a target than does a weak binding ligand. Tight and weak binding noncovalent complexes are in equilibrium with the free

10   ligand and free target. If a target is incubated with a mixture of two ligands, e.g., a tight binding and a weak binding ligand, an equilibrium will be established between the bound and unbound forms of each ligand with the binding site of the biological target. At

15   equilibrium, an equilibrium constant (binding constant) can be calculated and is used as a measure of the binding affinities of the ligands. Binding affinity is a measure of the attraction between a ligand and its target.

      A binding site is the specific region of a target

20   where a substrate or a ligand binds to form a complex. For example, an enzyme's active site is where catalysis takes place. In a structured RNA molecule, binding of a ligand at a binding site can result in the disruption of the transcription or translation processes. A ligand is

25   a small molecule that binds to a particular large molecule, a target molecule. Typically the target molecule is a large molecule, as for instance, a biological target such as a protein (enzyme) or a structured RNA or DNA.

30   A preferred target molecule is RNA particularly structured RNA. Structured RNA is a term that refers to definable, relatively local, secondary and tertiary structures such as hairpins, bulges, internal loops, junctions and pseudoknots. Structured RNA can have both

35   base paired and single stranded regions. RNA can be

divided into primary, secondary, and tertiary structures
and is defined similarly to proteins. Thus the primary
structure is the linear sequence. The secondary
structure reflects local intramolecular base pairing to
5   form stems and single stranded loops, bulges, and
junctions. The tertiary structure reflects the
interactions of secondary structural elements with each
other and with single stranded regions.

Mass spectrometry (MS) is a powerful analytical tool
10   for the study of molecular structure and interaction
between small and large molecules. The current state of
the art in MS is such that sub-femtomole quantities of
material can be readily analyzed to afford information
about the molecular contents of the sample. An accurate
15   assessment of the molecular weight of the material may be
quickly obtained, irrespective of whether the samples'
molecular weight is several hundred, or in excess of a
hundred thousand, atomic mass units or Daltons (Da). It
has now been found that mass spectrometry can elucidate
20   significant aspects of important biological molecules.
One reason for the utility of MS as an analytical tool is
the availability of a variety of different MS methods,
instruments, and techniques that can provide different
pieces of information about the samples.
25   A mass spectrometer analyzes charged molecular ions
and fragment ions from sample molecules. These ions and
fragment ions are then sorted based on their mass to
charge ratio (m/z). A mass spectrum is produced from the
abundance of these ions and fragment ions that is
30   characteristic of every compound. In the field of
biotechnology, mass spectrometry has been used to
determine the structure of a biomolecule, as for instance
determining the sequence of oligonucleotides, peptides,
and oligosaccharides.
35   In principle, mass spectrometers consist of at least

four parts: (1) an inlet system; (2) an ion source; (3) a
mass analyzer; and (4) a mass detector/ion-collection
system (Skoog, D.A. and West, D.M., Principles of
Instrumental Analysis, Saunders College, Philadelphia,

5   PA, **1980**, 477-485). The inlet system permits the sample
to be introduced into the ion source. Within the ion
source, molecules of the sample are converted into
gaseous ions. The most common methods for ionization are
electron impact (EI), electrospray ionization (ESI),

10   chemical ionization (CI) and matrix-assisted laser
desorption ionization (MALDI). A mass analyzer resolves
the ions based on mass-to-charge ratios. Mass analyzers
can be based on magnetic means (sector), time-of-flight,
quadrupole and Fourier transform mass spectrometry

15   (FTMS). A mass detector collects the ions as they pass
through the detector and records the signal. Each ion
source can potentially be combined with each type of mass
analyzer to generate a wide variety of mass
spectrometers.

20   Mass spectrometry ion sources are well known in the
art. Two commonly used ionization methods are
electrospray ionization (ESI) and matrix-assisted laser
desorption/ionization (MALDI) (Smith *et al.*, Anal. Chem.,
1990, *62*, 882-899; Snyder, in Biochemical and

25   Biotechnological Applications of Electrospray Ionization
Mass Spectrometry, American Chemical Society, Washington,
DC, **1996**; and Cole, in Electrospray Ionization Mass
Spectrometry: Fundamentals, Instrumentation, Wiley, New
York, **1997**).

30   ESI is a gentle ionization method that results in no
significant molecular fragmentation and preserves even
weakly bound complexes between biopolymers and other
molecules so that they are detected intact with mass
spectrometry. ESI produces highly charged droplets of

the sample being studied by gently nebulizing a solution
of the sample in a neutral solvent in the presence of a
very strong electrostatic field. This results in the
generation of highly charged droplets that shrink due to

5   evaporation of the neutral solvent and ultimately lead to
a "coulombic explosion" that affords multiply charged ions
of the sample material, typically via proton addition or
abstraction, under mild conditions.

       Electrospray ionization mass spectrometry (ESI-MS)

10   is particularly useful for very high molecular weight
biopolymers such as proteins and nucleic acids greater
than 10 kDa in mass, for it affords a distribution of
multiply-charged molecules of the sample biopolymer
without causing any significant amount of fragmentation.

15   The fact that several peaks are observed from one
sample, due to the formation of ions with different
charges, contributes to the accuracy of ESI-MS when
determining the molecular weight of the biopolymer
because each observed peak provides an independent means

20   for calculation of the molecular weight of the sample.
Averaging the multiple readings of molecular weight
obtained from a single ESI-mass spectrum affords an
estimate of molecular weight that is much more precise
than would be obtained if a single molecular ion peak

25   were to be provided by the mass spectrometer. Further
adding to the flexibility of ESI-MS is the capability of
obtaining measurements in either the positive or negative
ionization modes.

       ESI-MS has been used to study biochemical

30   interactions of biopolymers such as enzymes, proteins and
macromolecules such as oligonucleotides and nucleic acids
and carbohydrates and their interactions with their
ligands, receptors, substrates or inhibitors (Bowers *et
al.*, *Journal of Physical Chemistry*, **1996**, *100*, 12897–

12910; Burlingame *et al.*, *J. Anal. Chem.*, **1998**, *70*, 647R-716R; Biemann, *Ann. Rev. Biochem.*, **1992**, *61*, 977-1010; and Crain *et al.*, *Curr. Opin. Biotechnol.*, **1998**, *9*, 25-34). While interactions that lead to covalent

5   modification of biopolymers have been studied for some time, one of the most significant developments in the field has been the observation, under appropriate solution conditions and analyte concentrations, of specific non-covalently associated macromolecular

10   complexes that have been promoted into the gas-phase intact (Loo, *Mass Spectrometry Reviews*, **1997**, *16*, 1-23; Smith *et al.*, *Chemical Society Reviews*, **1997**, *26*, 191-202; Ens *et al.*, Standing and Chernushevich, Eds., *New Methods for the Study of Biomolecular Complexes,*

15   *Proceedings of the NATO Advanced Research Workshop, held 16-20 June 1996, in Alberta, Canada, in NATO ASI Ser., Ser. C, 1998, 510,* Kluwer, Dordrecht, Netherlands).

A variety of non-covalent complexes of biomolecules have been studied using ESI-MS and reported in the

20   literature (Loo, Bioconjugate Chemistry, **1995**, *6*, 644-665; Smith *et al.*, J. Biol. Mass Spectrom. **1993**, *22*, 493-501; Li *et al.*, J. Am. Chem. Soc., **1993**, *115*, 8409-8413). These include the peptide-protein complexes (Busman *et al.*, Rapid Commun. Mass Spectrom., **1994**, *8*, 211-216; Loo

25   *et al.*, Biol.. Mass Spectrom., **1994**, *23*, 6-12; Anderegg and Wagner, J. Am. Chem. Soc., **1995**, *117*, 1374-1377; Baczynskyj *et al.*, Rapid Commun. Mass Spectrom., **1994**, *8*, 280-286), interactions of polypeptides and metals (Loo *et al.*, J. Am. Soc. Mass Spectrom., **1994**, *5*, 959-965; Hu and

30   Loo, J. Mass Spectrom., **1995**, *30*, 1076-1079; Witkowska *et al.*, J. Am. Chem. Soc., **1995**, *117*, 3319-3324; Lane *et al.*, J. Cell Biol., **1994**, *125*, 929-943), and protein-small molecule complexes (Ganem and Henion, ChemTracts-

Org. Chem., **1993**, *6,* 1-22; Henion *et al.,* Ther. Drug
Monit., **1993**, *15,* 563-569; Ganguly *et al., Tetrahedron,*
1993, *49,* 7985-7996, Baca and Kent, J. Am. Chem. Soc.,
1992, *114,* 3992-3993). Further, the study of the
quaternary structure of multimeric proteins (Baca and
Kent, J. Am. Chem. Soc., **1992**, *114,* 3992-3993; Light-Wahl
et al., J. Am. Chem. Soc., **1994**, *116,* 5271-5278; Loo, J.
Mass Spectrom., **1995**, *30,* 180-183, Fitzgerald *et al.,*
Proc. Natl. Acad. Sci. USA, **1996**, *93,* 6851-6856), and of
nucleic acid complexes (Light-Wahl *et al.,* J. Am. Chem.
Soc., **1993**, *115,* 803-804; Gale *et al.,* J. Am. Chem. Soc.,
1994, *116,* 6027-6028; Goodlett *et al.,* Biol. Mass
Spectrom., **1993**, *22,* 181-183; Ganem *et al.,* Tet. Lett.,
1993, *34,* 1445-1448; Doctycz *et al.,* Anal. Chem., **1994**,
66, 3416-3422; Bayer *et al.,* Anal. Chem., **1994**, *66,* 3858-
3863; Greig *et al.,* J. Am. Chem. Soc., **1995**, *117,* 10765-
766), protein-DNA complexes (Cheng *et al., Proc. Natl.*
Acad. Sci. U.S.A., **1996**, *93,* 7022-7027), multimeric DNA
complexes (Griffey *et al., Proc. SPIE-Int. Soc. Opt.*
Eng., **1997**, *2985,* 82-86), and DNA-drug complexes (Gale *et*
al., JACS, **1994**, *116,* 6027-6028) are known in the
literature.

ESI-MS has also been effectively used for the
determination of binding constants of non-covalent
macromolecular complexes such as those between proteins
and ligands, enzymes and inhibitors, and proteins and
nucleic acids. The use of ESI-MS to determine the
dissociation constants (K_D) for oligonucleotide-bovine
serum albumin (BSA) complexes have been reported (Greig
et al., J. Am. Chem. Soc., **1995**, *117,* 10765-10766). The
K_D values determined by ESI-MS were reported to match
solution K_D values obtained using capillary
electrophoresis.

ESI-MS measurements of enzyme-ligand mixtures under competitive binding conditions in solution afforded gas-phase ion abundances that correlated with measured solution-phase dissociation constants (K_D) (Cheng *et al.*, *JACS*, **1995**, *117*, 8859-8860). The binding affinities of a 256-member library of modified benzenesulfonamide inhibitors to carbonic anhydrase were ranked. The levels of free and bound ligands and substrates were quantified directly from their relative abundances as measured by ESI-MS and these measurements were used to quantitatively determine molecular dissociation constants that agree with solution measurements. The relative ion abundance of non-covalent complexes formed between D- and L-tripeptides and vancomycin group antibiotics were also used to measure solution binding constants (Jorgensen *et al.*, *Anal. Chem.*, **1998**, *70*, 4427-4432).

ESI techniques have found application for the rapid and straightforward determination of the molecular weight of certain biomolecules (Feng and Konishi, Anal. Chem., **1992**, *64*, 2090-2095; Nelson *et al.*, Rapid Commun. Mass Spectrom., **1994**, *8*, 627-631). These techniques have been used to confirm the identity and integrity of certain biomolecules such as peptides, proteins, oligonucleotides, nucleic acids, glycoproteins, oligosaccharides and carbohydrates. Further, these MS techniques have found biochemical applications in the detection and identification of post-translational modifications on proteins. Verification of DNA and RNA sequences that are less than 100 bases in length has also been accomplished using ESI with FTMS to measure the molecular weight of the nucleic acids (Little *et al*, Proc. Natl. Acad. Sci. USA, **1995**, *92*, 2318-2322).

While data generated and conclusions reached from ESI-MS studies for weak non-covalent interactions

generally reflect, to some extent, the nature of the
interaction found in the solution-phase, it has been
pointed out in the literature that control experiments
are necessary to rule out the possibility of ubiquitous
5   non-specific interactions (Smith and Light-Wahl, Biol.
Mass Spectrom., **1993**, *22*, 493-501). The use of ESI-MS
has been applied to study multimeric proteins because the
gentleness of the electrospray/desorption process allows
weakly-bound complexes, held together by hydrogen
10   bonding, hydrophobic and/or ionic interactions, to remain
intact upon transfer to the gas phase. The literature
shows that not only do ESI-MS data from gas-phase studies
reflect the non-covalent interactions found in solution,
but that the strength of such interactions may also be
15   determined. The binding constants for the interaction of
various peptide inhibitors to src SH2 domain protein, as
determined by ESI-MS, were found to be consistent with
their measured solution phase binding constants (Loo *et
al.*, Proc. 43rd ASMS Conf. on Mass Spectrom. and Allied
20   Topics, **1995**). ESI-MS has also been used to generate
Scatchard plots for measuring the binding constants of
vancomycin antibiotics with tripeptide ligands (Lim *et
al.*, J. Mass Spectrom., **1995**, *30*, 708-714).

. Similar experiments have been performed to study
25   non-covalent interactions of nucleic acids. ESI-MS has
been applied to study the non-covalent interactions of
nucleic acids and proteins. Stoichiometry of interaction
and the sites of interaction have been ascertained for
nucleic acid-protein interactions (Jensen *et al.*, Rapid
30   Commun. Mass Spectrom., **1993**, 7, 496-501; Jensen *et al.*,
42nd ASMS Conf. on Mass Spectrom. and Allied Topics, **1994**,
923). The sites of interaction are typically determined
by proteolysis of either the non-covalent or covalently
crosslinked complex (Jensen *et al.*, Rapid Commun. Mass

Spectrom., **1993**, 7, 496-501; Jensen *et al.*, 42nd ASMS

Conf. on Mass Spectrom. and Allied Topics, **1994**, 923;

Cohen *et al.*, Protein Sci., **1995**, *4*, 1088-1099).

Comparison of the mass spectra with those generated from

5   proteolysis of the protein alone provides information

about cleavage site accessibility or protection in the

nucleic acid-protein complex and, therefore, information

about the portions of these biopolymers that interact in

the complex.

10   Fourier transform ion cyclotron resonance mass

spectrometry (FT-ICR MS) is an especially useful

analytical technique because of its ability to resolve

very small mass differences to make mass measurements

with a combination of accuracy and resolution that is

15   superior to other MS detection techniques, in connection

with ESI ionization (Amster, J. Mass Spectrom., **1996**, *31*,

1325-1337, Marshall *et al.*, *Mass Spectrom. Rev.*, **1998**,

17, 1-35).   FT-ICR MS may be used to obtain high

resolution mass spectra of ions generated by any of the

20   other ionization techniques.   The basis for FT-ICR MS is

ion cyclotron motion, which is the result of the

interaction of an ion with a unidirectional magnetic

field. The mass-to-charge ratio of an ion (m/q or m/z) is

determined by a FT-ICR MS instrument by measuring the

25   cyclotron frequency of the ion.

The insensitivity of the cyclotron frequency to the

kinetic energy of an ion is one of the fundamental

reasons for the very high resolution achievable with FT-

ICR MS.   Each small molecule with a unique elemental

30   composition carries an intrinsic mass label corresponding

to its *exact* molecular mass, identifying closely related

library members bound to a macromolecular target requires

only a measurement of exact molecular mass.   The target

and potential ligands do not require radio labeling,

fluorescent tagging, or deconvolution via single compound
re-synthesis. Furthermore, adjustment of the
concentration of ligand and target allows ESI-MS assays
to be run in a parallel format under competitive or non-
5   competitive binding conditions. Signals can be detected
from complexes with dissociation constants ranging from <
10 nM to ~100 mM. FT-ICR MS is an excellent detector in
conventional or tandem mass spectrometry, for the
analysis of ions generated by a variety of different
10   ionization methods including ESI, or product ions
resulting from collisionally activated dissociation.

FT-ICR MS, like ion trap and quadrupole mass
analyzers, allows selection of an ion that may actually
be a weak non-covalent complex of a large biomolecule
15   with another molecule (Marshall and Grosshans, Anal.
Chem., **1991**, *63*, A215-A229; Beu *et al.*, J. Am. Soc. Mass
Spectrom., **1993**, *4*, 566-577; Winger *et al.*, J. Am. Soc.
Mass Spectrom., **1993**, *4*, 566-577; Huang and Henion, Anal.
Chem., **1991**, *63*, 732-739), or hyphenated techniques such
20   as LC-MS (Bruins *et al.*, Anal. Chem., **1987**, *59*, 2642-
2646; Huang and Henion, J. Am. Soc. Mass Spectrom., **1990**,
1, 158-65; Huang and Henion, Anal. Chem., **1991**, *63*, 732-
739) and CE-MS experiments (Cai and Henion, J.
Chromatogr., **1995**, *703*, 667-692). FTICR-MS has also been
25   applied to the study of ion-molecule reaction pathways
and kinetics.

The use of ESI-FT-ICR mass spectrometry as a method
to determine the structure and relative binding constants
for a mixture of competitive inhibitors of the enzyme
30   carbonic anhydrase has been reported (Cheng *et al.*, J.
Am. Chem. Soc., **1995**, *117*, 8859-8860). Using a single
ESI-FT-ICR MS experiment these researchers were able to
ascertain the relative binding constants for the
noncovalent interactions between inhibitors and the

enzyme by measuring the relative abundances of the ions of these noncovalent complexes. Further, the K_Ds so determined for these compounds paralleled their known binding constants in solution. The method was also

5   capable of identifying the structures of tight binding ligands from small mixtures of inhibitors based on the high-resolution capabilities and multistep dissociation mass spectrometry afforded by the FT-ICR technique. A related study (Gao *et al.*, J. Med. Chem., **1996**, *39*, 1949-

10   55) reports the use of ESI-FT-ICR MS to screen libraries of soluble peptides in a search for tight binding inhibitors of carbonic anhydrase II. Simultaneous identification of the structure of a tight binding peptide inhibitor and determination of its binding

15   constant was performed. The binding affinities determined from mass spectral ion abundance were found to correlate well with those determined in solution experiments. Heretofore, the applicability of this technique to drug discovery efforts is limited by the

20   lack of information generated with regards to sites and mode of such non-covalent interactions between a protein and ligands.

Electrospray ionization (ESI) has found wide acceptance in the field of analytical mass spectrometry

25   since it is a gentle ionization method which produces multiply charged ions from large molecules with little or no fragmentation and promotes them into the gas phase for direct analysis by mass spectrometry. ESI sources operate in a continuous mode with flow rates ranging from

30   < 25nL/min to 1000 µL/min. The continuous nature of the ion source is well suited for mass spectrometers which employ the m/z scanning, such as quadrupole and sector instruments, as their coupling constitutes a continuous ion source feeding in a nearly continuous mass analyzer.

As used in this invention the electrospray ionization source may have any of the standard configurations including but not limited to Z-spray, microspray, off-axis spray or pneumatically assisted electrospray. All of

5   these can be used in conjunction with or without additional countercurrent drying gas. Further the mass spectrometer can include a gated ion storage device for effecting thermolysis of test mixtures.

When the solvated ions generated from electrospray

10   ionization conditions are introduced into the mass spectrometer, the ions are subsequently desolvated in an evaporation chamber and are collected in a rf multi-pole ion reservoir (ion reservoir). A gas pressure around the ion reservoir is reduced to 10^{-3} - 10^{-6} torr by vacuum

15   pumping. The ion reservoir is preferably driven at a frequency that captures the ions of interest and the ensemble of ions are then transported into the mass analyzer by removing or reversing the electric field generated by gate electrodes on either side of the ion

20   reservoir. Mass analysis of the reacted or dissociated ions are then performed. Any type of mass analyzers can be used in effecting the methods and process of the invention. These include, but are not limited to, quadrupole, quadrupole ion trap, linear quadrupole, time-

25   of-flight, FT-ICR and hybrid mass analyzers. A preferred mass analyzer is a FT-ICR mass analyzer.

Seen in Figure 1 is a schematic representation of a mass spectrometer. A review of the mass spectrometer will facilitate understanding of the invention as it

30   includes various component parts that may be included in one or more of the various types of different mass spectrometers. The spectrometer **10** includes a vacuum chamber **12** that is segmented into a first chamber **14** and a second chamber **16**. The mass spectrometer **10** is shown

as an electrospray mass spectrometer. A metallic micro-electrospray emitter capillary **18** having an electrode **20** is positioned adjacent to the vacuum chamber **12**. The electrode/metallic capillary serves as an ion emitter.

5 The capillary **18** is positioned on an X-Y manipulator for movement in two planes.

Adjacent to the capillary **18** and extending from the vacuum chamber **16** is an evaporative chamber **22** having a further capillary **24** extending axially along its length.

10 The X-Y manipulator allows for precise positioning of the capillary **18** with respect to the capillary **24**. A plume of ions carried in a solvent is emitted from the emitter capillary **18** towards the evaporator capillary **24**. The evaporator capillary **24** serves as an inlet to the

15 interior of vacuum chamber **12** for that portion of the plume directly in line with the evaporator capillary **24**.

Within the first chamber **14** is a skimmer cone **26**. This skimmer cone **26** serves as a lens element. In line with the skimmer cone **26** is an ion reservoir **28**. A port

20 **30** having a valve is connected to a conventional first vacuum source (not shown) for reducing the atmospheric pressure in the first chamber **14** to create a vacuum in that chamber. Separating chambers **14** and **16** is a gate electrode **32**.

25 The ion reservoir **28** can be one of various reservoirs such as a hexapole reservoir. Ions, carried in a solvent, are introduced into chamber **14** via the evaporator capillary **24**. Solvent is evaporated from the ions within the interior of capillary **24** of the

30 evaporator chamber **22**. Ions travel through skimmer cone **26** towards the electrode **32**. By virtue of their charge and a charge placed on the electrode **32** the ions can be held in the reservoir.

The electrode **32** includes an opening. Ions are released from the ion reservoir **28** by modifying the potential on the electrode **32**. They then can pass through the opening into the second vacuum chamber **16**
5   towards a mass analyzer **34**. For use in FT-ICR, positioned with respect to the analyzer **34** is a magnet (not shown). The second vacuum chamber **16** includes port **36** having a valve. As with valve **30** in chamber **14**, this valve **36** is attached to an appropriate vacuum pump for
10   creating a vacuum in chamber **16**. Chamber **16** may further include a window or lens that is positioned in line with a laser. The laser can be used to excite ions in either the mass analyzer **34** or the ion reservoir.

In effecting the methods and processes of the
15   invention, a set of compounds are probed against a target molecule, using the mass spectrometer, to identify those compounds, i.e., ligands, from the set of compounds that are "weak" binders with respect to the target molecule. For the purposes of this invention "weak" binding is
20   defined as binding in the millimolar (mM) range. Typically ligands will have a binding affinity in the range of 0.2 to 10 nM. As opposed to other techniques, the mass spectrometer will not fail to detect these weak mM interactions. Ligands having preferred binding
25   characteristics with respect to a target molecule are selected. After selection, the binding mode of the ligands is determined by re-screening mixtures of ligands against the target molecule. Re-screening is effected by simultaneously exposing the target molecule against a
30   small set of two more ligands. As a result of this screening, ligands that can not bind at overlapping sites, competitive binding, are differentiated from those that can bind at remote sites simultaneously, concurrent binding, and those that can bind in a way that traps one

compound, cooperative binding as well as those having "mixed" binding modes.

Ligands having selected binding characteristics are identified and their structure activity relationship (SAR) with respect to target binding is probed using the mass spectrometer. Two or more ligands can be joined by concatenation into new structural configurations to create a new ligand that will have improved binding characteristics or properties. Thus starting from small, rigid ligands that bind with weak affinity, more complex molecules that bind to specific target molecules with high affinity can be identified using mass spectrometry. This is effected using the mass spectrometer as the primary tool and does not involve extensive chemical synthesis or extensive molecular modeling.

Concatenation can be effected based on empirical or computational predictions. Thus concatenation will yield either new synthetic chemical ligands having new properties or *in silico* virtual ligands. In conjunction with molecular modeling tools, the virtual ligands can be used to identify probable binding locations on the target molecule.

In concatenating ligands together using the methods and processes of the invention, two ligands that have mM (millimolar) affinities might be joined and yield a concatenated ligand that might have nM affinity (nanomolar). While we do not wish to be bound by theory, we presently believe this result has multiple contributing factors. There can be a gain in intrinsic binding energy, i.e., loss of translational entropy, when both fragments always bind at the same time. Proper geometry for both fragments can result in a favorable enthalpy of interaction, i.e., no loss of binding enthalpy. Fewer degrees of freedom resulting from two

fragments being linked through bonds with limited
rotation will result in a loss of rotational entropy that
equals a gain in binding energy. And there can be some
energy gain (enthalpy and entropy) from desolvation of
the target and the ligand fragments. The net result can
be a 10^3 to a 10^6 improvement in binding affinity, i.e., a
4-6 kcal/mol gain in binding energy.

Newly synthesized concatenated ligand molecules,
which retain the best conformations and locations of the
ligand fragments with respect to the target, can be re-
probed using the mass spectrometer to ascertain the
binding characteristics of the new molecule. Repeated
iteration of the process and methods of the invention can
improve the binding affinity of these new molecules. The
newly synthesized concatenated ligand molecules can also
be screened using a functional assay that involves the
target.

The target molecule can be any target of interest.
Preferred as targets are molecules of biological interest
especially RNA, proteins, RNA-DNA duplexes, DNA duplexes,
polysaccharides, phospholipids and glycolipids. A
particularly preferred target molecule is RNA. As
practiced herein, the target molecule can, itself, be a
fragment of a larger molecule, as for instance, RNA that
is a fragment of a larger RNA. Particularly preferred as
a target molecule is RNA especially RNA that is a
fragment of a larger RNA. A further preferred target
molecule is double stranded DNA targeted with ligands
that are transcription factors.

The initial weak binding ligands can be selected
from various sources including, but not limited to,
collection libraries of diverse compounds. These
include, but are not limited to, historical repositories
of compounds, collections of natural products,
collections of drug substances, collections of

intermediates produced in forming drug substances,
collections of dye stuffs, commercial collections of
chemical substances or combinatorial libraries of related
compounds. Many universities and pharmaceutical

5   companies maintain historical repositories of all
compounds synthesized. These can include drugs
substances that have or have not been screened for
biological activity, intermediates used in the
preparation of such drug substances and derivatives of

10   such drug substances. A typical pharmaceutical company
might have millions of such repository samples. Other
collections of compounds include collections of natural
occurring compounds or derivatives of such natural
occurring compounds. Irrespective of the origin of the

15   compounds, the compound collections can be categorized by
size, structure, function or other various parameters.

      Commercial chemical supply houses also have
collections of compounds that are suitable for screening
against target molecules. Again these might be

20   categorized by various parameter that can be useful in
selecting sets of compounds for screening against a
target molecule to identify weak binding ligands for that
target molecule. Other ligand molecule candidates might
be specifically synthesized to include one or more

25   features. One preferred method to assemble a group of
compounds useful for selecting binding ligands is by
effecting a combinatorial synthesis of a group of related
compounds using various methods that are available in the
art of combinatorial chemistry.   Irrespective of the

30   source of the ligands, i.e., from a collection or
specifically synthesized according to define criteria,
the ligands will contain various motifs, i.e., stacking,
electrostatic and H-bonding, that contribute to the weak
binding of the ligands with the target.

35   The collections of compound for consideration as

32

ligands for target molecules, typically categorized by
size, structure or function, can be assembled as a
library or set of compounds having from 2 to about
100,000 or less members. In a first preferred group of
5   compounds selected for consideration as ligands for a
target molecule each member of the group would be
selected to independently have a molecular mass less than
about 1000 Daltons and fewer than 15 rotatable bonds. In
a more preferred group of compounds, each member of the
10   group will be selected to independently have a molecular
mass less than about 600 Daltons and fewer than 8
rotatable bonds. In a more preferred group of compounds
each of member of the group will be selected to
independently have a molecular mass less than about 200
15   Daltons, have fewer than 4 rotatable bonds or no more
than one sulfur, phosphorous or halogen atom. A
particularly useful solvent for use in screening
potential ligands for an RNA target is dimethylsulfoxide.
In a particularly preferred method of the invention, the
20   potential ligands are selected as compounds having at
least 20 mM solubility in dimethylsulfoxide.

In screening a compound set for potential binding
ligands, sample preparation and certain basic operations
of the mass spectrometer are optimized to preserve the
25   weak non-covalent complexes formed between ligands and
the target molecule. These include extra care in
desalting the target molecule as well as a general
reduction of the temperature of the desolvation capillary
compared to the temperature that would be used if the
30   only interest was in analyzing the target molecule
itself. Also the voltage potential across the capillary
exit and the first skimmer cone, i.e., lens element, is
optimized to ensure good desolvation. A further
consideration is selection of the buffer concentration
35   and solvent to insure good solvation.

33

In selecting potential weak binding ligands for a target molecule a standard ligand is used as a reference ligand for that target. Various standard ligands will be used for different targets. In one sense, these standard

5   ligands can be thought of as ion thermometers. With any. target molecule, the standard compounds will typically be selected such that its has a binding affinity, as measured as a dissociation constant, i.e., Kd, of the order of nanomolar to about 100 millimolar for its target

10   molecule. A more preferred range would be from 10 to 50 mM with 50 mM binding affinity for the target molecule being the most preferred.

For use with RNA or DNA targets we have found ammonium (from acetate, chloride, borate or other salts),

15   primary amines (including by not limited to alkyl amines such as methylamine and ethylamine), secondary amines (including but not limited to dialkylamines such as dimethylamine and diethylamine), tertiary amines (including by not limited to trialkyl amines such as

20   triethylamine, trimethylamine and dimethylethyl amine), amino acids (including but not limited to glycine, alanine, tryptophan and serine) and nitrogen containing heterocycles (including but not limited to imidazole, triazole, triazine, pyrimidine and pyridine) are

25   particularly useful. These standard ligands will typically have a binding affinity, as measured as a dissociation constant, i.e., Kd, of the order of nanomolar to about 100 millimolar for the RNA or DNA target. Ammonium is particularly useful for RNA since it

30   has a binding affinity for RNA, as measure by its dissociation constant, i.e., Kd, of about 50mM.

Other standard ligands will be used for other target molecules. For use with protein target molecules, esters such as formate, acetate and propionate, phosphates,

35   borates, amino acids and nitrogen containing heterocycles

(including but not limited to imidazole, triazole, triazine, pyrimidine and pyridine) are particularly useful. As with the above described RNA and DNA target molecules, for protein target molecules as well as for other target molecules, the standard ligands will typically have a binding affinity, as measured as a dissociation constant, i.e., Kd, of the order of nanomolar to about 100 millimolar for the target.

By selecting the binding affinity for the standard ligand as described, the operating performance conditions of the mass spectrometer are adjusted such that the signal strength of the standard ligand to that of the target molecule of from 1% to about 30% of the signal strength of unbound target. One or more of the candidate ligands from a set of compounds is next screened with a mixture of the target molecule and the standard ligand. Those candidate ligands having weak affinities can be identified by the presence of a signal that is greater than the background noise of the mass spectrometer. By adjustment of the operating conditions of the mass spectrometer using the standard ligands, non-binding ligands are not detected by the mass spectrometer.

The candidate ligands can be screened one at a time or in sets. A typical set would have from 2 to 10 members. A more preferred set has from 4 to 8 members.

The compound set is screened for members that form non-covalent complexes with the target molecule using the mass spectrometer. The relative abundances and stoichiometries of the non-covalent complexes with the target molecule are measured from the integrated ion intensities. These results can be stored in a relational database that is cross-indexed to the structure of the compounds.

For a typical RNA target, the RNA is selected as an RNA molecule have from about 10 to about 200 nucleotides.

This RNA can be an isolated or purified fragment of a larger RNA or it can be a synthetic RNA. Such synthetic RNA can be a mimic of a natural RNA. A more preferred RNA target molecule would have from about 15 to about 100

5   nucleotides. The RNA can have both secondary and ternary structure.

Having derived a set of ligands that bind to the target molecule, in one embodiment of the invention, simple derivative of these ligands are made by modifying

10   the ligand. These modifications include modification by addition of methyl, amino, nitro, hydroxyl, bromo, thio groups or other small substituent group or derivatives where the composition and size of rings and side chains have been varied. These derivatives can then be screened

15   as above to obtain SAR information and to optimize the binding affinity with the target.

Depending on the size of the compound collection used above, from 2 to 10,000 compounds may form complexes with the target. These compounds are pooled into groups

20   of 4-10 and screened again as a mixture against the target as before. Since all of the compounds have been shown previously to bind to the target, three possible changes in the relative ion abundances are observed in the mass spectrometry assay. If two compounds bind at

25   the same site, the ion abundance of the target complex for the weaker binder will be decreased through competition for target binding with the higher affinity binder (competitive binding). If two compounds can bind at distinct sites, signals will be observed from the

30   respective binary complexes with the target and from a ternary complex where both compounds bind to the target simultaneously (concurrent binders). If the binding of one compound enhances the binding of a second compound, the ion abundance from the ternary complex will be

35   enhanced relative to the ion abundances from the

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respective binary complexes (cooperative binding. If the ratio of the relative ion abundances is greater than 1, the binding is considered to be cooperative. These ratios of relative ion abundances are calculated and can

5   be stored in a database for all compounds that bind to the target.

Compounds that bind concurrently are further analyzed. Derivatives of concurrent binders can be prepared with addition of an added moiety, including but

10   not limited to methyl, ethyl, isopropyl, amino, methylamino, dimethylamino, trifluoromethyl, methoxy, thiomethyl or phenyl at different positions around the original compound that binds. These derivatives can be re-screened as a mixture with compounds that bound

15   concurrently to the starting compound. If the additional methyl, ethyl, isopropyl, or phenyl moiety occupies space that the concurrent binder occupied, the two compounds will bind competitively. Observation of this change in the mode of binding using the mass spectrometer indicates

20   the two molecules are spatially proximate as a result of the chemical modification. Correlation of the change in binding mode with the size and position of the chemical modification can be used as a "molecular ruler" to measure the distance between two compounds on the surface

25   of the RNA. Compounds that bind in a cooperative or competitive mode do so by binding in close proximity on the target surface. Locations where addition of a moiety has no effect on the binding mode are potential sites of covalent attachment between the two molecules. This

30   information can be used in conjunction with molecular modeling of the target-ligand complex to generate a pharmacophore map of the chemical groups that bind to the target surface.

In some cases, a 3-dimensional working model of the

35   target structure may be available based on NMR or

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chemical and enzymatic probing data. These 3-D models of the target can be used with computational programs such as MCSS (MSI, San Diego) or QXP (Thistlesolft, Groton, CT) to locate the possible sites of binding with the

5   ligand. MCSS, QCP and similar programs perform a Monte Carlo-based search for sites where the ligand can bind, and rank order the sites based on a scoring scheme. The scoring scheme calculates hydrophobic, hydrogen-bonding, and electrostatic interactions between the ligand and

10   target. The small molecules may bind at many locations along the surface of the target. However, there are some locations that are preferred. These calculations can be performed for molecules that bind competitively or cooperatively, and favorable binding conformations whose

15   proximity is based on the "molecular ruler" as described above can be identified.

     In one embodiment of the invention, the QXP program is used to search all interaction space around a RNA target molecule and to cluster the results. From the

20   clustered results the highest probability, low-energy binding sites for binding ligands is identified. All the interaction space around the RNA target is searched for proximate binding sites between ligands. The distances between the ligands are measured to obtain the lengths of

25   linkers required to connect functional group sites on the ligands for best scaffold binding. The search also is used to insure that the lowest energy conformation retains the best binding contacts.

     In conjunction with the developers of QXP, the UNIX

30   version of the QXP program designed to run on a SGI computer having 128 processors was ported to a LINUX version that runs on a PC platform having 56 processors. This resulted in an advantage in maximizing the price to performance ratio of the hardware. The computationally

35   intensive nature of identifying global energy minimum for

a combinatorial library of small molecule, typically with 8 - 12 rotatable bonds, bound to the receptor is particularly well suited to the "distributed computing" method. The compound library is divided into the number of available computational resources and thus the docking calculations are run in "parallel". This method exploits the available CPU cycles over a cluster of extremely fast PC boxes networked together in a system commonly referred to as a Beowulf-class cluster. Beowulf-class clusters are described by E. Wilson in Chemical & Engineering News (2000, 78(2):27-31) The PC platform used included 16 PCs, dual Intel pentium II 450 MHz processors, 256 MB RAM and 6.4 GB disk and 12 PCs, dual Intel pentium II 400 MHz processors, 256 MB RAM and 6.4 GB disk totaling 56 processors. A benchmark calculation using 350MHz Pentium II processors indicated, in terms of speed, that PC boxes clustered together as described would outperform a R5000 SGI O2 machine.

The same result is reported to be accomplished using the MCSS software, i.e., MCSS/HOOK. As reported by its manufacture, MSI, San Diego, CA, for proteins, MCSS/HOOK characterizes an active site's ability to bind ligands using energetics calculated via CHARMm. Strongly bound ligands are linked together automatically to provide de novo suggestions for drug candidates. The software is reported to provide a systematic, comprehensive approach to ligand development and de novo ligand design that result in synthetically feasible molecules. Using libraries of functional groups and molecules, MCSS is reported to systematically searches for energetically feasible binding sites in a protein. HOOK is reported to then systematically searches a database for skeletons which logically might connect these binding sites in the presence of the protein. HOOK attempts to link multiple

functional groups with molecular templates taken from the its database. The results are potential compounds that are consistent with the geometry and chemistry of the binding site.

5   An embodiment of the invention relates to methods of determining the relative interaction of ligands that bind to a target substrate. The exposure of a target substrate to a mixture of two or more ligands allows for the formation of noncovalent complexes. Analysis of the

10   mixture by mass spectrometry enables the identification of the complexes formed, the relative affinities of the ligands and ultimately the type of interaction between the ligands for the target. In the simplest situation, two ligands known to bind to a target are mixed together

15   and screened by mass spectrometry leading to three conditional relationships; competitive, concurrent and cooperative binding.

Competitive binding

20   Ligands bind competitively for a target when the binding of one ligand prevents the binding of the other ligand is the result of the ligands binding to the target at the same location. In this situation, the mixture contains an equilibrium of two binary complexes, one of

25   which being one ligand bound to the target and the other being the other ligand bound to the target. The ligand having the greater affinity for the target will predominate and thus have higher signal intensity for its binary complex with the target compared to the other

30   ligand. Competitive binding interaction between two ligands is determined according to methods of the invention by analyzing the mixture by mass-spectrometry to detect the presence or lack of signal corresponding to a ternary complex where both ligands are bound to the

target at the same time. The lack of signal for a
ternary complex indicates a competitive binding
interaction between the two ligands while the presence of
the signal indicates a non-competitive interaction.

5   Accordingly, in an aspect of the present invention,
there is provided a method for determining the relative
interaction between at least two ligands with respect to
a target substrate. In practicing this method an amount
of each of the ligands is mixed with an amount of the
10   target substrate to form a mixture. This mixture is
analyzed by mass spectrometry to determine the presence
or absence of a ternary complex corresponding to the
simultaneous adduction of two of the ligands with the
target substrate. The absence of the ternary complex
15   indicates that binding of the ligands to the target
substrate is competitive and the presence of the ternary
complex indicates that binding of the ligands to the
target substrate is other than competitive.

          The above method for determining a competitive
20   binding interaction of two ligands is exemplified in
figure 3 wherein 70µM of a small molecule Ibis-326732 (4-
amino-2-piperidin-4-ylbenzimidazole) was added to a
solution of 100µM glucosamine and 5µM of a 27 nucleotide
fragment of bacterial 16S ribosomal RNA incorporating the
25   A-site. The mass-spectrum trace for the mixture lacks an
intensity signal for a ternary complex of the two ligands
Ibis-326732 and glucosamine simultaneously bound to the
target 16S RNA. This indicates that the two ligands are
competitive binders for this target i.e. bind to the same
30   site. Further, a comparison of the ion abundance of the
two binary complexes at approximately 1762 and 1770 m/z
indicates that Ibis-326732 binds to the target RNA with
greater affinity than glucosamine.

Concurrent binding

Ligands bind concurrently when the binding of one
ligand to the target is unaffected by the binding of the
other and is a consequence of the ligands binding to the
target at distinct sites. In this situation, a mixture
containing two concurrent binding ligands will have an
equilibrium of two binary complexes, one being first
ligand bound to the target and the other being the second
ligand bound to the target as well as a ternary complex
of both ligands bound to the target and unbound target
substrate. The ligand having the greater affinity for
the target will have higher signal intensity for its
binary complex with the target compared to the other
ligand. Concurrent binding interaction between two
ligands is determined according to methods of the
invention by analyzing the mixture by mass-spectrometry
and comparing the ratios of the ion abundance of the
complexes. Particularly, the absolute ion abundance of
the ternary complex (TL1L2) is compared to the relative
ion abundance of the binary complexes (TL1 and TL2) which
contribute to the formation of the ternary complex with
respect to the unbound target (TL1 X TL2 / T). Since
there are two binary complexes contributing the formation
of the ternary complex, the comparison is with the sum of
the two contributing binary complexes i.e. TL1 X TL2/T +
TL2 X TL1/T. If the absolute ion abundance of the
ternary complex is equal to the sum of the relative ion
abundance of the contributing binary complexes, then the
two ligands concurrently bind to the target substrate.
Expressed another way, a pair of ligands are concurrent
binders for a target if in either of the following
equivalent formulae the value of y is equal to zero:

$$y \;=\; TL1L2 \;-\; TL1 \;X\; \frac{TL2}{T} \;-\; TL2 \;X\; \frac{TL1}{T}$$

or

$$y = TL1L2 - 2 \times \frac{TL1 \times TL2}{T}$$

The above method for determining a concurrent binding interaction of two ligands is exemplified in figure 4 wherein 3,5-diamino-1,2,4-triazole (DT) and 2-deoxystreptamine (2-DOS) are both ligands for target RNA (a 27-mer fragment of ribosomal RNA comprising the 16S A-site). The mass-spectrum trace shows intensity signals for a ternary complex at approximately 1778 m/z for both ligands bound to the target 16S RNA, a binary complex at about 1758 m/z for 2-DOS bound to 16S RNA, a binary complex at 1746 m/z for DT bound to 16S RNA and another signal at about 1727 m/z for 16S RNA unbound by either ligand. The relative ion abundance of the ternary complex (16S+2-DOS+DT) with respect to the unbound 16S target RNA (16S) is equal, within limits of error, to the sum of the relative ion abundance of the contributing binary complex ((16S+DT) X (16S+2-DOS)) with respect to the unbound target (16S) and the contributing binary complex ((16S+2-DOS) + (16S+DT)) with respect to the unbound target (16S). Expressed in a simplified form of the formula:

$$y \approx (16S+2\text{-}DOS+DT) - 2 \times (16S+2\text{-}DOS) \times (16S+DT) / 16S$$

This indicates a concurrent binding interaction between the two ligands, 2-DOS and DT, for the target 16S RNA. Further, a comparison of the ion abundance of the two binary complexes indicates that 2-DOS has greater binding affinity for the target RNA than DT.

Cooperative binding

Ligands bind cooperatively when the binding of one ligand to the target enhances the binding of the other, i.e. more of the first ligand will bind to the target in the presence of the second ligand than in its absence. Cooperatively binding ligands may bind to their target at distinct locations. In a mixture containing two cooperatively binding ligands there will be an equilibrium of two binary complexes, a ternary complex and unbound target. The ternary complex is a simultaneous adduction of both ligands to the target. One of the binary complexes is complex of the first ligand bound to the target and the other binary complex is that of the second ligand bound to the target. The ligand having the greater affinity for the target will demonstrate a higher signal intensity for its binary complex with the target compared to the other ligand. Cooperative binding interaction between two ligands is determined according to methods of the invention by analyzing the mixture by mass-spectrometry and comparing the absolute ion abundance of the ternary complex to the sum of the relative ion abundance of the binary complexes contributing to the formation of the ternary complex in the same manner as for concurrent binders. However, in the instance of cooperative binding ligands, the relative ion abundance of the ternary complex (TL1L2/T) is greater than the sum of the relative ion abundances of the contributing binary complexes. Expressed another way, a pair of ligands are concurrent binders for a target if in either of the following equivalent formulae the value of y is greater than zero:

$$y \;=\; TL1L2 \;-\; TL1 \times \frac{TL2}{T} \;-\; TL2 \times \frac{TL1}{T}$$

44

or

$$y = TL1L2 - 2 \times \frac{TL1 \times TL2}{T}$$

5

Mixed binding

10   Another scenario can arise when comparing the ion abundances, that is, when the ternary ion abundance is less than the sum of the relative abundances of the contributing binary complexes (i.e. y of the above formulae is less than zero). This indicates a more complex binding situation where there is a combination of interactions resulting from a competitive interaction

15   between the ligands while at the same time another non-competitive interaction (cooperative or concurrent) is also occurring. Stated another way, this indicates a mixed binding mode arising when either or both ligands have more than one binding site on the target that may be

20   detected by a mass-spectrum signal for the multiply bound target. Complex binding interaction of two ligands includes competitive/cooperative, competitive/concurrent, cooperative/concurrent, competitive/cooperative/concurrent or further

25   combinations thereof.

    A mixture in which two ligands have both competitive and concurrent binding interactions will exhibit a mass-spec signal for a ternary complex whereas a mixture having only a competitive interaction will exhibit no

30   such signal. A mixture in which two ligands exhibit both a competitive and cooperative interaction will exhibit a mass-spec signal for the ternary complex and the absolute ion abundance for the ternary complex (TL1L2) will be greater than the sum of the relative ion abundance for

35   the contributing binary complexes when the cooperative

interaction is predominant. Conversely, the absolute
ternary abundance will be less when the competitive
interaction is stronger than the cooperative interaction.
When there is both competitive and concurrent binding

5   interaction, the absolute ternary ion abundance will be
less than the sum of the relative ion abundances for the
contributing binary complexes and greater when there is
both cooperative and concurrent binding interaction.

10   A further embodiment of the invention includes
methods for determining the relative proximity and
orientation of binding sites for a first ligand and a
second ligand on a target substrate. The target
substrate is exposed to a mixture of the second ligand

15   and at least one derivative compound of the first ligand.
Derivative compounds of the first ligand are derivative
structures that include the first ligand and have at
least one substituent group pendent from the first
ligand. The mixture is analyzed by mass spectrometry to

20   identify those first ligand derivatives that inhibits the
binding of the second ligand to the target substrate. In
this embodiment, the method of determining the mode of
binding interaction previously discussed may be used to
determine the spatial proximity of ligand binding sites

25   on a target. For example, the knowledge that two ligands
are concurrent binders indicates that they have separate
and distinct binding sites. In order to determine the
distance between these two binding sites, derivatives of
one of the ligands are prepared and mixed with the other

30   ligand and the target. The derivatives of the first
ligand will have the core chemical structure of the
ligand but will also have substituents pending from the
structure, the substituents having a diversity of lengths
and attachment points to the structure.

35   A ligand derivative that inhibits the binding of the

second ligand to the target, i.e. a derivative that is competitive with the second ligand, provides insight into the proximity and orientation of the binding sites relative to each other. A competitive derivative is identified by mass-spec analysis of the mixture and its particular substituent and attachment point on the parent ligand structure is determined. The point of attachment of the substituent indicates the relative orientation while the length of the substituent indicates the relative proximity of the binding sites. In this way the substituent group serves as a molecular ruler and compass.

An efficient manner of performing the method is by employing combinatorial chemistry techniques to create a library of ligand derivatives having great diversity in substituents. Suitable substituent groups include but are not limited to alkyl (e.g. methyl, ethyl, propyl), alkenyl (e.g. allyl), alkynyl (e.g. propynyl), alkoxy (e.g. methoxy, ethoxy), alkoxycarbonyl, acyl, acyloxy, aryl (e.g. phenyl), aralkyl, hydroxyl, hydroxylamino, keto (=O) amino, alkylamino (e.g. methylamino), mercapto, thioalkyl (e.g. thiomethyl, thioethyl), halogen (e.g. chloro, bromo), nitro, haloalkyl (e.g. trifluoromethyl), phosphorous, phosphate, sulfur and sulfate. In further embodiment of the invention, the invention includes a screening method for determining compounds having binding affinity to a target substrate. A mixture of the ligands and the target substrate are analyzed by mass spectrometry. First and second ligand that bind to the target substrate are identified. These first and second ligands are concatenated to form a third ligand having greater binding affinity for the target substrate than either first or second ligand. In this embodiment of the invention, ligands are identified using mass spectrometry methods described herein and are concatenated or linked

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together to form a new ligand incorporating the chemical
structure responsible for binding of the two parent
ligands to the target. The new concatenated ligand will
have greater binding affinity for the target than either

5   of the two parent ligands. An example of this is
illustrated in examples 4 and 5 and figures 6-8 where
mass-spec analysis of a library of amide compounds
revealed two having binding affinity for a fragment of
bacterial 16S ribosomal RNA. The two ligands (IBIS-

10   271583 and IBIS-326611) both incorporated a piperazine
moiety and a concatenated compound of the two ligands was
prepared having a common piperazine moiety from which the
remainder of the ligand structures depend. The
concatenated compound (IBIS-326645) is shown in figure 8

15   to bind the target 16S RNA fragment with greater affinity
(52.4% of the target) than either of the two parent
ligands in figures 6 and 7 (27.8% and 14.7%
respectively). In a preferred embodiment, the new
concatenated ligand comprises the chemical structure of

20   the first and second ligands linked together by a linking
group. Suitable linking groups are well known in the art
and depend upon the chemical structure of the ligands and
are preferably linked to atoms of the ligand molecule not
directly involved in binding to the target.

25   Linking groups are selected that generally are of a
length that results in a reduction in entropy of the
ligand target system. Typically a linker will have a
length of about 15 Angstroms, preferably less than about
10 Angstroms and more preferably less than 5 Angstroms.

30   Preferred linking groups include but are not limited to a
direct covalent bond, alkylene (e.g. methylene,
ethylene), alkenylene, alkynylene, arylene, ether (e.g.
alkylethers), alkylene-esters, thioether, alkylene-
thioesters, aminoalkylene (e.g. aminomethylene),

35   amine, thioalkylene and heterocycles (e.g. pyrimidines,

48

piperizine and aralkylene).

An example of the above method is shown in figures 5
through 7. In separate mixtures, 200μM of three ligands
IBIS-326611 ((2S)-2-amino-3-hydroxy-1-piperazinylpropan-
1-one), IBIS-326645 (5-methyl-1-(2-oxo-2-
piperazinylethyl)-1,3-dihydropyrimidine-2,4-dione) and a
concatenated compound thereof, IBIS-271583 (1-{2-[(3R)-4-
((2S)-2-amino-3-hydroxypropanoyl)-3-methylpiperazinyl]-2-
oxoethyl}-5-methyl-1,3-dihydropyrimidine-2,4-dione) are
each mixed with 5μM of target 16S RNA fragment and
analyzed by mass spectrometry. IBIS-326611 is shown in
figure 5 to form a binary complex having an ion abundance
27.8% that of the unbound 16S RNA while IBIS-326645 in
figure 6 forms a binary complex having an ion abundance
14.7% that of the unbound 16S RNA. The concatenated
compound IBIS-271483 on the other hand forms a binary
complex having 52.4% ion abundance relative to unbound
16S RNA, and therefor has greater affinity for the target
16S RNA than either of the parent compounds.

New concatenated ligands may be screened in the same
manner as were the parent ligands, and the affinities of
those that bind may be measured through titration of the
ligand concentration. The binding location of the new
molecule on the target may be determined using a mass
spectrometry-based protection assay, infrared multiphoton
dissociation, NMR, X-ray crystallography, AFM force
microcopy and other known techniques. Suitable
concatenated ligands having improved affinity may then be
screened in functional assays to demonstrate a biological
effect appropriate for a drug molecule. If the
biological activity is insufficient, the molecules may be
iterated through the process additional times.

In a preferred embodiment the linking group is
chosen based on the relative orientation and proximity of
the ligand binding sites by exposing the target substrate

to a mixture of the second ligand and a plurality of derivative compounds of the first ligand wherein the first ligand derivatives comprising the chemical structure of the first ligand and at least one

5   substituent group pending therefrom. The mixture is analyzed by mass spectrometry to identify a first ligand derivative that inhibits the binding of said second ligand to the target substrate. In this method, mass spectrometry is used to infer the local environments of

10   ligands. The footprint of one or more of the binding ligands may be increased through addition of substituents such as methyl, ethyl, amino, methylamino, methoxy, ethoxy, thiomethyl, thioethyl, bromo, nitro, chloro, trifluoromethyl and phenyl groups at different positions.

15   This allows a SAR series to be constructed (either virtually or in vitro) for each individual ligand. For example, a methyl group may be added to the first ligand and it is found by the mass-spec screening that the methyl group does not affect the binding of the second

20   ligand. This suggests that a methyl group may be an appropriate point to use for ligation with the second ligand. For example, it was found that first and second ligands bind cooperatively to a target and that a methyl derivative of the first ligand retains the cooperative

25   binding with the second ligand. This indicates that point of attachment of the methyl group on the first ligand may be a suitable point on that ligand for linking to the second ligand. In the instance where the binding sites of the first and second ligand overlap, a concatenated

30   compound comprising a fusion of the two chemical structures that are responsible for binding to the target will have greater affinity to the target than either first or second ligand.

       Alternatively, the orientation and proximity of the

35   binding sites may be determined by molecular modeling

techniques, i.e., *in silico*, using programs such as MCSS
(LeClerk, 1999) and others that virtually reproduce
stacking, hydrogen bonding and electrostatic contacts
with the target. Preferably, orientation and proximity of

5   the binding sites is determined by a combination of
molecular modeling and the methods employing derivatized
ligands in an iterative process wherein each technique
provides information useful in performing the other. For
example, molecular modeling may predict the orientation

10   of a ligand at its binding site and give insight into the
position at which a substituent or linking group may be
attached to the ligand. Other techniques may also be
used separately or in combination with those mentioned
such as X-ray crystallography which provides 3-

15   dimensional orientation and location when bound to its
target. Another technique available for determining
orientation and proximity of ligands at their binding
site for designing linking groups is by NMR. A
particular NMR method for determining orientation and

20   proximity is described in patent application WO97/18469
which claims priority from USSN 08/558,644 (filed 14
November 1995) and 08/678,903 (filed 12 July 1996) each
incorporated herein by reference. In this NMR method a
target molecule is labeled with ^{15}N and analyzed by ^{15}N/^{1}H

25   NMR correlation spectroscopy when bound by the ligands.
This method is particularly useful for targets that are
easily labeled with ^{15}N such as proteins and peptide.

EXAMPLES

General

All MS experiments were performed by using an Apex
II 70e ESI-FT-ICR MS (Bruker Daltonics, Billerica, MA)
with an actively shielded 7 tesla superconducting magnet.
RNA solutions were prepared in 50 mM NH_4OAc (pH 7), mixed
with 10% isopropanol to aid desolvation, and infused at a
rate of 1.5 μL/min by using a syringe pump. Ions were
formed in a modified electrospray source (Analytica,
Branford, CT) by using an off-axis grounded electrospray
probe positioned ca.
1.5 cm from the metallized terminus of the glass
desolvation capillary biased at 5,000 V. A countercurrent
flow of dry oxygen gas heated to 150°C was used to assist
in the desolvation process.. Ions were accumulated in an
external ion reservoir comprised of a radio
frequency-only hexapole, a skimmer cone, and an auxiliary
electrode for 1,000 ms before transfer into the trapped
ion cell for mass analysis. Each spectrum was the result
of the co-addition of 64 transients comprised of 524,288
data points acquired over a 217,391-kHz bandwidth,
resulting in a 1.2-sec detection interval. All aspects of
pulse sequence control, data acquisition, and
postacquisition processing were performed by using a
Bruker Daltonics data station running XMASS Version 4.0
on a Silicon Graphics (Mountain View, CA) R5000
computer.

Example 1

*Mass spectrometry-based selection of compounds with
affinity for RNA.*

RNA binding ligands are selected from a set of
compounds using mass spectrometry. The RNA use for the
target molecule is an RNA whose electrospray ionization

properties have been optimized in conjunction with optimization of the electrospray ionization and desolvation conditions. A set of compounds that contains members with molecular mass less than 200, 3 or fewer

5   rotatable bonds, no more than one sulfur, phosphorous, or halogen atom, and at least 20 mM solubility in dimethylsulfoxide is used. A 50 µM stock solution of the RNA is purified, and dialyzed to remove sodium and potassium ions.

10   The compound set is pooled into mixtures of 8 members, each present at 1-10 mM in DMSO. A collection of these mixtures is diluted 1:50 into an aqueous solution containing 50-150 mM ammonium acetate buffer at pH 7.0, 1-5 µM RNA target, and 10-50% isopropanol, ethanol, or

15   methanol to create the screening sample. The aqueous solution contains 100 µM each of 8 compounds, 50 mM ammonium acetate, 5 µM RNA target, and 25% isopropanol. These screening samples are arrayed in a 96-well microtiter plate, or added to individual vials for

20   queuing into an automated robotic liquid hander under computer control by the mass spectrometer.

       ' The source voltage potentials are adjusted to give stable electrospray ionization by monitoring the ion abundance of the free RNA. The temperature of the

25   desolvation capillary is next reduced incrementally and the voltage potential between the capillary and the first skimmer lens element of the mass spectrometer is adjusted until adducts of ammonia with the RNA can be observed. If available on the mass spectrometers, the partial gas

30   pressure beyond the desolvation capillary is adjusted by throttling the pumping speed. This gas pressure may also be altered to optimize the ion abundance and observation of the ammonium ion adducts. After instrument performance has been optimized, the voltage potential

35   between the capillary and skimmer lens is increase to

53

reduce the abundance of the ion from the monoammonium-RNA
complex to ~10% of the abundance of the ion from the RNA.
These instrument parameters are used for detection of
complexes between the RNA and compound set.

5   The compound set is screened for members that form
non-covalent complexes with the RNA. The relative
abundances and stoichiometries of the non-covalent
complexes with the RNA are measured from the integrated
ion intensities, and the results are stored in a
10   relational database cross-indexed to the structure of the
compounds.

Figure 2 shows the resulting spectrum obtained after
adjustment of operating performance conditions of the
mass spectrometer for detection of weak affinity
15   complexes. Free target RNA is seen at 1726.7 m/z in the
spectrum. Ions associated with adducts of ammonium with
the RNA target can be observed and are easily
differentiated from sodium ion adducts based on the
combined molecular mass of the ammonium/RNA adducts.
20   Ions associated with an adduct of a triazole ligand (2-
amino-4-benzylthio-1,2,4-triazole) are also seen. The
RNA target is present at a concentration 5 micromolar and
the triazole ligand at a concentration of 100 micromolar
and the relative abundances of the ion peaks are
25   normalized to that of the target RNA.

Example 2

*Chemical optimization of compounds that form complexes
with the RNA target.*

30   In a second step, compounds are obtained with
structures derived from those selected in Example 1.
These compounds may be simple derivatives with additional
methyl, amino, or hydroxyl groups, or derivatives where
the composition and size of rings and side chains have

been varied. These derivatives are screened as in
Example 1 to obtain SAR information and to optimize the
binding affinity with the RNA target.

5   **Example 3**

   *Determination of the mode of binding for compounds
forming complexes with the RNA target.*

         In the compound collection used in Example 1, those
compounds that formed complexes with the RNA target are
10   pooled into groups of 4-10 and screened again as a
mixture against the RNA target as outlined in Example 1.
   Since all of the compounds have been shown previously to
bind to the RNA, three possible changes in the relative
ion abundance are observed in the mass spectrometry
15   assay. If two compounds bind at the same site, the ion
abundance of the RNA complex for the weaker binder will
be decreased through competition for RNA binding with the
higher affinity binder (competitive binding). An example
is presented in Figure 3, where the ion abundance from a
20   glucosamine-RNA complex is reduced as glucosamine is
displaced from the RNA by addition of a benzimidazole
compound. If two compounds can bind at distinct sites,
signals will be observed from the respective binary
complexes with the RNA and from the ternary complex where
25   both compounds bind to the RNA simultaneously (concurrent
binders). If the binding of one compound enhances the
binding of a second compound, the ion abundance from the
ternary complex will be enhanced relative to the ion
abundance from the respective binary complexes
30   (cooperative binding). An example of cooperative binding
between 2-deoxystreptamine (2-DOS) and 3,5-
diaminotriazole (3,5-DT) is presented in Figure 4. The
relative ion abundance from the secondary complex for
3,5-DT to the free RNA is measured, as is the relative

ion abundance from the ternary complex between 3,5-DT, 2-DOS, and RNA and the binary complex. If the ratio of the relative ion abundance is greater than 1, the binding is considered to be cooperative. The ratios of relative ion

5   abundance are calculated and stored in a database for all compounds that bind to this RNA.

Example 4

Amide Library Synthesis - General Procedures

10   Operations involving resin were carried out in a Quest 210 automated synthesizer (Argonaut Technologies, San Carlos, CA). HPLC/MS spectra were obtained on a HP1100 MSD system (Hewlett-Packard, Palo Alto, CA) equipped with a SEDEX (Sedere) evaporative light

15   scattering detector (ELSD). A 4.6 x 50 mm Zorbax XDB-C18 reversed phase column (Hewlett-Packard, Palo Alto, CA) was operated using a linear gradient of 5% A to 100% B over 4 min at 2 mL/min flow rate (A = 10 mM aqueous ammonium acetate + 1% v/v acetic acid, B = 10 mM ammonium

20   acetate in 95:5 v/v acetonitrile/water + 1% v/v acetic acid. The flow was split 3:1 after the column, with 0.5 mL/min flowing to the MSD mass detector, and 1.5 mL/min flowing to the ELSD detector. Quantitation was based on integration of the ELSD peak corresponding to product,

25   which was identified by the corresponding mass spectrum of the eluting peak. ^{1}H NMR spectra for all compounds were recorded either at 399.94 MHz on a Varian Unity 400 NMR spectrometer or at 199.975 MHz on a Varian Gemini 200 NMR spectrometer.

30

General Procedure for Synthesis of Secondary Amine Resins: Preparation of AG-MB-benzylamine resin

2-methoxy-4-alkoxy-benzaldehyde PEG-PS resin (ArgoGel-MB-CHO, Argonaut Technologies, San Carlos, CA,

10 g, 0.4 mmole/g) was slurried in 30 ml dry
trimethylorthoformate (TMOF). Benzylamine (0.52 ml, 4.8
mmole) was added and the slurry swirled gently on a
shaker table under dry nitrogen overnight. A solution of
5   40 ml dry methanol, acetic acid (0.46 ml, 8.0 mmole) and
borane-pyridine complex (1.0 ml, 8.0 mmole) was added,
and the slurry swirled overnight. The resin was filtered,
and washed several times with methanol, DMF, CH_2Cl_2, and
finally methanol. Gel-phase NMR showed complete
10   conversion from the aldehyde to secondary benzylamine
derivative. Gel-phase ^{13}C NMR (C_6D_6) δ 40.9, 48.1, 53.0,
54.8, 67.7, 70.9 (PEG linker), 99.5, 104.7, 121.3, 127.0,
127.8 (poly-styrene beads), 128.5, 130.5, 141.2, 159.0,
159.8.
15   The supports AG-MB-cyclohexylamine and AG-MB-methylamine,
were similarly prepared using cyclohexyl and methylamine
(used as a methanol solution available from Aldrich),
respectively. The following are the resins employed and
the resulting amine functionality of the library
20   compounds.

resin	amine functionality
1,2-diaminoethane-PS	1,2-diaminoethane
2-OH-1,3-diaminopropane-PS	2-OH-1,3-diaminopropane
AG-MB-benzylamine	benzylamine
AG-MB-cyclohexylamine	cyclohexylamine
AG-MB-methylamine	methylamine
AG-Rink-NH-Fmoc	amino
PS-trityl-piperazine	piperazine

General Procedure for Synthesis of Amide Motifs
The desired carboxylic acid (1 eq.) was suspended in dry
DMF (5 mL/mmole), and HATU (Perseptive Biosystems, 1 eq.)
and collidine (3 eq.) was added. The suspension was

stirred for 15 min, and if a suspension still existed, diisopropylethylamine (1 eq.) was added, and stirring continued. At this point all acids were in solution. This 0.2 M (5 eq. per eq. of amine on the resin) solution

5   of activated acid was added to the appropriate resin containing a primary or secondary amine, and the mixture was agitated overnight at 65 °C. The resins were either purchased from Novabiochem, Argonaut Technologies, or prepared *via* the general procedure. The mixture was

10   filtered, and the resin washed with DMF (3x), MeOH (3x), CH$_2$Cl$_2$ (3x), DMF (3x) and CH$_2$Cl$_2$ (3x) and dried with a flow of inert gas. To the resulting resin, trifluoroacetic acid (7 mL/g dry resin) containing 5% v/v triisopropylsilane was added, and the suspension agitated

15   for 4 h. The mixture was filtered, and the resin washed with trifluoroacetic acid (3x). The combined filtrates were concentrated to afford the desired products. The products were characterized by HPLC/MS and were generally sufficiently pure for testing.

20   The following are the carboxylic acids each of which were coupled with each of the resin bound amines listed above. The corresponding amide functionality of the resulting library compounds are listed thereafter.

25   carboxylic acid
     (R)-(-)-2,2-dimethyl-5-oxo-1,3-dioxolane-4-acetic acid
     (S)-(+)-2,2-dimethyl-5-oxo-1,3-dioxolane-4-acetic acid
     2,3-dihydroxyquinoxaline-6-carboxylic acid
     2-N-Bhoc-guanine-1-acetic acid

30   4-N-Bhoc-cytosine-1-acetic acid
     6-N-Bhoc-adenine-1-acetic acid
     bis(BOC-3,5-diaminobenzoic acid)
     BOC-3-ABZ-OH
     BOC-benzimidazole-5-carboxylic acid

35   BOC-glycine

BOC-imidazole-4-carboxylic acid

BOC-isonipecotic acid

BOC-SER(tBu)-OH

FMOC-3-amino-1,2,4-triazole-5-carboxylic acid

5 nalidixic acid

N-BOC-L-homoserine

orotic acid

t-butoxyacetic acid

thymine-1-acetic acid

10

amide functionality

(R)-3-hydroxy-3-carboxypropionyl

(S)-3-hydroxy-3-carboxypropionyl

2,3-dihydroxyquinoxaline-6-carboxyl

15 guanine-1-acetyl

cytosine-1-acetyl

adenine-1-acetyl

3,5-diaminobenzoyl

3-aminobenzoyl

20 5-carboxy-benzimidazole

1-aminoacetyl

imidazole-4-carboxyl

isonipecotyl

(2S)-2-amino-3-hydroxypropionyl

25 3-amino-1,2,4-triazole-5-carboxyl

nalidixoyl

(2S)-2-amino-4-hydroxybutyryl

orotyl

hydroxyacetyl

30 thymine-1-acetyl

Example 5

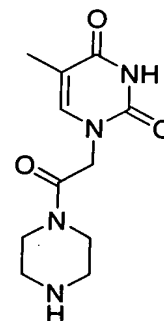   *(2S)-2-Amino-3-hydroxy-1-piperazinylpropan-1-one*

According to the general procedure, the title compound was prepared using PS-trityl-piperazine resin (Novabiochem) and BOC-(tBu)-Serine (Bachem): HPLC/MS M+H 174 fnd., (0.25 min, 100%)

5   *Thymine-1-acetylpiperazine*

According to the general procedure, the title compound was prepared using PS-trityl-piperazine resin (Novabiochem) and thymine-1-acetic acid (Aldrich): HPLC/MS M+H = 253 fnd., (0.29 min, 100%).

10
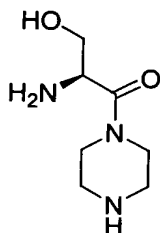
(2S)-2-amino-3-hydroxy-1-piperazinylpropan-1-one   thymine-1-acetylpiperazine

15   *1-{2-[(3R)-4-((2S)-2-Amino-3-hydroxypropanoyl)-3-methylpiperazinyl]-2-oxoethyl}-5-methyl-1,3-dihydropyrimidine-2,4-dione.*

HATU (1.1 g, 2.7 mmol) and DIEA (4.7 mL, 27 mmol) were added sequentially to a solution of Boc-Ser(tBu)-OH (0.71
20   g, 2.7 mmol) in DMF (10 mL). The mixture was stirred at room temperature for about 30 min then was added to a solution of (R)-(-)-2-methylpiperazine (0.3 g, 3 mmol) in DMF (5 mL). The mixture was stirred for 12 h and was diluted with a mixture of sat. NaHCO$_3$/EtOAc (200 mL, v/v,
25   50:50). The aqueous layer was extracted with more EtOAc (2 X 30 mL). The combined organic layer was dried (Na$_2$SO$_4$),

filtered, and concentrated in vacuo to give a colorless oily residue, which was used in the next step without purification.

HATU (0.38 g, 1.0 mmol) and 2,4,6-collidine (0.73 mL, 5.5 mmol) were added sequentially to a solution of thymine-1-acetic acid (0.19 g, 1 mmol) in DMF (5 mL). The mixture was stirred at room temperature for about 30 min then was added to a solution of the residue prepared above in DMF (5 mL). The mixture was stirred for 12 h and was diluted with a mixture of sat. $NaHCO_3$/EtOAc (100 mL, v/v, 50:50). The aqueous layer was extracted with more EtOAc (2 X 10 mL). The combined organic layer was dried (Na_2SO_4), filtered, and concentrated in vacuo to give a colorless oily residue. Purification of the residue by flash column chromatography (gradient elution 3-5% MeOH/CH_2CL_2) provided N-BOC-O-t-butyl protected derivative (38 mg, 8% yield in two step): TLC (R_f = 0.4; 10% MeOH/CH_2Cl_2); ^{13}CNMR (DMSO-d_6) δ 169.8, 165.4, 164.4, 155.2, 151.0, 142.2, 107.9, 78.2, 72.7, 61.5, 50.3, 48.2, 45.1, 28.1, 27.1, 11.8; HRMS (MALDI) m/z 532.2736 (M+Na)$^+$ ($C_{24}H_{39}N_5O_7$ requires 532.2747).

A solution of the protected derivative (23.4 mg, 0.046 mmol) in concentrated aqueous HCl (2 mL) was stirred at room temperature for 12 h. The reaction mixture was evaporated to give the title compound (20 mg, quantitative yield). ^{13}C NMR (CD$_3$OD) δ 167.3, 167.0, 153.2, 143.9, 111.0, 73.6, 72.4, 62.2, 60.8, 54.4, 47.1, 46.5, 43.8, 12.3.

Example 6

2-Deoxy-1,3-diazido-4-[(5-bromo-3-nitro-1,2,4-triazolyl)methyl]-5,6-di-O-acetylstreptamine

Dry hydrogen chloride is passed through a solution of 2-deoxy-1,3-diazido-5,6-di-*O*-acetylstreptamine (296 mg, 1 mmole, prepared according to the method of Wong et. al., *J. Am. Chem. Soc.* **1999**, *121*, 6527-6541) and paraformaldehyde (45 mg, 1.5 mmole) in dichlorethane at 0° C for 6 h. Solid $CaCl_2$ is added, the mixture filtered, then concentrated *in vacuo*. The syrup is azeotroped three times with dry acetonitrile to provide the chloromethyl derivative. Separately, a suspension of 5-bromo-3-nitro-1,2,4-triazole (386 mg, 2 mmole) is stirred with sodium hydride (60% w/w, 80 mg, 2 mmole) for 0.5 h in acetonitrile (20 mL). This suspension is then added directly to the chloromethyl derivative, and the mixture stirred overnight at room temperature. Water and ethyl acetate were added, the organic layer collected, dried over magnesium sulfate, concentrated, and chromatographed (20% ethyl acetate/hexanes) to provide the title compound.

2-Deoxy-1,3-diazido-4-[(5-amino-3-nitro-1,2,4-triazolyl)methyl]streptamine

2-Deoxy-1,3-diazido-4-[(5-bromo-3-nitro-1,2,4-triazolyl)methyl]-5,6-di-*O*-acetylstreptamine is dissolved in 3:1 dioxane/28% aqueous ammonia, and the solution stirred at 60°C in a sealed vessel overnight. The

5   solvent is removed, and the residue chromatographed (10% methanol/chloroform) to provide the title compound.

2-Deoxy-4-[(3,5-diamino-1,2,4-triazolyl)methyl]streptamine

2-Deoxy-1,3-diazido-4-[(5-amino-3-nitro-1,2,4-

10   triazolyl)methyl]streptamine is dissolved in ethanol, and hydrogenated over 10% palladium on carbon catalyst at 50 psi with shaking for 72 h. The mixture was filtered through celite, and the solvent removed to afford the title compound.

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